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A model for the reaction mechanism of the transglutaminase 3 enzyme

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Abbreviations: fTG, fish TGase; fXIIIa, blood clotting factor XIIIa; TGase, transglutaminase

Abstract

Transglutaminase enzymes (TGases) catalyze the calcium dependent formation of an isopeptide bond between protein-bound glutamine and lysine substrates. Previously we have shown that activated TGase 3 acquires two additional calcium ions at site two and three. The calcium ion at site three results in the opening of a channel. At this site, the channel opening and closing could modulate, depending on which metal is bound. Here we propose that the front of the channel could be used by the two substrates for enzyme reaction. We propose that the glutamine substrate is directed from Trp236 into the enzyme, shown by molecular docking. Then a lysine substrate approaches the opened active site to engage Trp327, leading to formation of the isopeptide bond. Further, direct comparisons of the structures of TGase 3 with other TGases have allowed us to identify several residues that might potentially be involved in generic and specific recognition of the glutamine and lysine substrates.

Keywords: calcium ions; protein structure; residue specificity; TGase; TGase mechanism; X-ray crystallography

Introduction

Transglutaminases (TGase; protein-glutamine: amine γ-glutamyl-transferase) are a family of calcium-dependent acyl-transfer enzymes that are widely expressed in biota (Folk and Chung, 1985; Greenberg *et al*., 1991; Melino *et al*., 1998; Melino *et al*., 2000; Fesus and Piacentini, 2002). Each of the eight active human TGase enzyme isoforms can activate a protein-bound Gln residue to form a thiol-acyl enzyme intermediate, which is attacked by a second nucleophilic substrate to accomplish the two-step reaction. The reaction leads to the formation of an isopeptide bond between two proteins and the covalent incorporation of polyamine into protein (Folk and Chung, 1985; Greenberg *et al*., 1991). The nucleophile may be: water, so that the activated Gln residue is deamidated to a Glu; a polyamine, so that a mono-substituted adduct or bi-substituted cross-link is formed; an alcohol, particularly the ω-hydroxyl group of certain long-chain ceramides expressed in mammalian epidermis, to form an ester; and perhaps most commonly, an ε-NH2 group of a protein bound Lys residue, resulting in the formation of an N^{ε}-(γ-glutamyl)lysine isopeptide cross-link (Nemes *et al*., 1999; Nemes *et al*., 2000). This stabilizes macromolecular protein complexes. Typically, TGases recognize the generic sequence motif Gln-Gln*-Val for the first step of the reaction (where Gln* represents the targeted residue), although different isoforms display specificity as to which substrates bearing the motif may approach the enzyme (Nemes *et al*., 1999). Anecdotal data suggest less specificity for Lys substrates (Tarcsa *et al*., 1998; Candi *et al*., 1999; Nemes *et al*., 1999).

The three dimensional structures of four TGases have now been reported, i.e. human factor XIIIa (hfXIIIa) (Yee *et al*., 1994), TGase 2 (Liu *et al*., 2002), TGase 3 enzymes (Ahvazi *et al*., 2002; 2003), and a fish enzyme (fTG, equivalent to mammalian TGase 2, Noguchi *et al*., 2001). All consist of four domains that are similar in organization and fold (Figure 1a, b for TGase 3): the amino terminal β-sandwich domain; the catalytic core domain which contains the conserved active site triad of Cys272, His330 and Asp353 (using TGase 3 residue numbers); the β-barrel 1 domain; and the β-barrel 2 domain at the carboxy terminus. FXIIIa and TGase 3 are zymogens that require proteolytic cleavage at different sites for activation, whereas most other such enzymes are constitutively active.

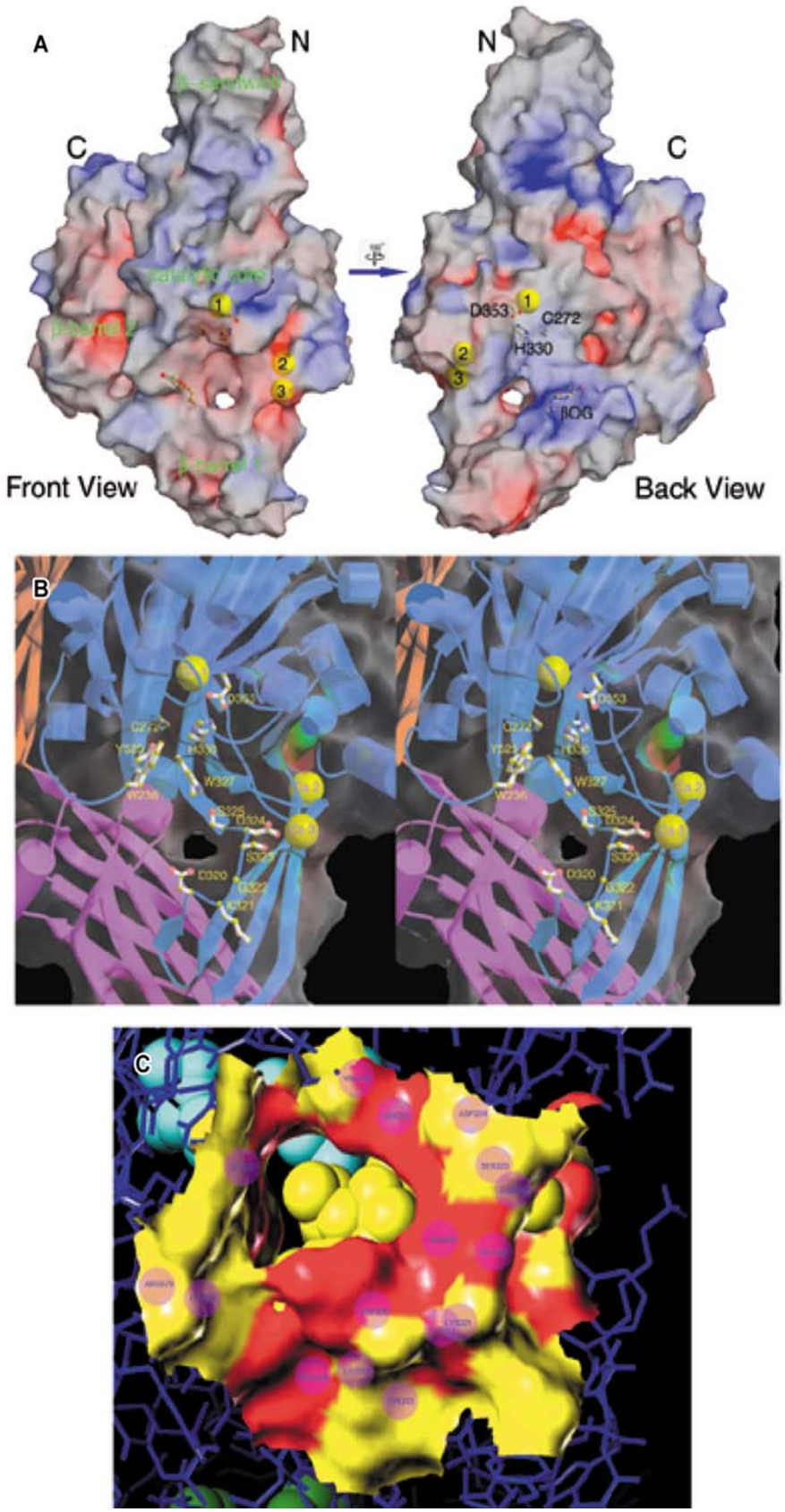

Figure 1. (A) The solved structure of activated human TGase 3 in presence of Ca^{2+} ions. The four domains and Ca^{2+} ions are shown. The electrostatic potential has been mapped onto the surface plan from -12.0 kT (deep red, acidic) to +12.0 kT (deep blue, basic). The β-octylglucoside molecule is shown as ball and stick. The catalytic triad residues Cys272, His330, and Asp353 and location of the three Ca^{2+} ions are shown in yellow. (B) Stereo views of the electrostatic surface potential (black transparent) show that the active site triad residues Cys272, His330 and Asp353 are buried and inaccessible. (C) The connolly surface channel is shown in the active form. The movement of the loop bearing residues Asp320-Ser325 opens the channel in the activated TGase 3, and exposes the side chains of the Trp236 and Trp327 residues.

One intriguing feature of TGases is their rather slow rate of reaction. When measured using either an artificial substrate such as methylated casein or the known favored substrate loricrin, the fastest rate of reaction of activated TGase 1 or 3 enzymes that has been measured is about 1,000 pmol of incorporated putrescine/hour/pmol of enzyme, which corresponds to one reaction cycle per 3-4 seconds (Kim *et al*., 1994; Candi *et al*., 1998). TGase 2 is comparably slow (Kim *et al*., 1994). However, the structural features that determine substrate specificity and this very modest rate of reaction remain largely unexplored. In addition, the solved TGase structures reveal the presence of three unusual *cis* peptide bonds located in the catalytic core domain in motifs that flank the active site triad residues. Typically such bonds are energetically unfavorable and are thought to be present in proteins because they are required for some aspect of the reaction mechanism cycle and/or confer special stability (Weiss *et al*., 1998; Jabs *et al*., 1999).

One of the most enigmatic aspects of catalysis by the TGases is that all are Ca^{2+} ion dependent for both *in vivo* (Folk and Chung, 1985) and *in vitro* reactions (Fox *et al*., 1999; Ahvazi *et al*., 2002; Ahvazi *et al*., 2003), although few data are currently available on how or why their dependence on Ca^{2+} metal ions is necessary. For example, the solved X-ray structures of the zymogen forms of hfXIIIa with or without a single Ca^{2+} ion do not reveal any structural changes (Yee *et al*., 1994; Yee *et al*., 1996; Fox *et al*., 1999). Furthermore, the structures of the fTG (Noguchi *et al*., 2001) and human TGase 2 (Liu *et al*., 2002) enzymes do not show any bound metal Ca^{2+} ion. In our comparison of the zymogen and activated TGase 3 enzyme we found that the zymogen possesses one tightly bound Ca^{2+} ion (PDB 1L9M) in site one but the proteolyzed form becomes active only after the exothermic binding of two more Ca^{2+} ions (PDB 1L9N) in sites two and three (Ahvazi *et al*., 2002). Notably, the local environment of the metal ion binding sites change upon Ca^{2+} ion chelation. A loop at the 'front' of the enzyme moves about 9 Å so that the side chain of Asp324 can coordinate with the Ca^{2+} ion in site three, and this opens a channel that passes through the activated enzyme just below the buried active site (Figure 1b and 1c; Ahvazi *et al*., 2002; Ahvazi *et al*., 2003). Thus the binding of these Ca^{2+} ions makes changes in the enzyme structure, presumably by allowing appropriate substrates to approach for reaction. Furthermore, we have recently shown by X-ray crystallography that the channel opening could be manipulated and controlled by intracellular cation levels such that the replacement of Ca^{2+} ion with Mg^{2+} results in the channel closing and inactivation of the enzyme (Ahvazi *et al*., 2003). Based on detailed comparison of current structural information on the zymogen and the activated forms of TGase 3, as well as molecular modeling of Gln and Lys substrates, we present here a new model for its mechanism of action that may offer clues as to how TGase 3 specificity is determined.

Extant Concepts for TGase Reaction Mechanism

In the available X-ray structures of TGase 3 (Ahvazi *et al*., 2002; Ahvazi *et al*., 2003), the catalytic triad active residues, including Cys272 at the active site, are buried in the hydrophobic interior of the enzyme. The sulfhydryl group of Cys272 forms an intimate thiolate-imidazolium ion pair with His330. The imino nitrogen atom of the His330 ring forms a hydrogen bond with the terminal oxygen atom of Asp353. In addition, the hydroxyl of Tyr525 is within hydrogen bonding distance of Cys272 and Trp236 and is located at the loop of the sequence motif Ile523-Asn526 of the β-barrel domain 1 that occludes the entrance to the active site (Figure 2a). These bonds must be cleaved and structural motifs near the enzyme's surface must be moved to allow substrates to approach the active site to effect reaction. Also, there is evidence that two tryptophan residues (Trp236 and Trp327), the indole rings of which are buried near the surface, are intimately involved in the enzyme reaction mechanism by forming an oxyanion intermediate first with the glutamine substrate and then with the lysine substrate, in order to form the isopeptide cross-link bond (Pedersen *et al*., 1994). Thus one early suggestion for a possible TGase enzyme mechanism is predicted that substrates should approach from the front side of the enzyme, dislodge some portion of the protein surface to expose the two Trp residues and break the hydrogen bonds masking the active site Cys residue (Yee *et al*., 1994; Noguchi *et al*., 2001). The importance of these two Trp residues has recently been confirmed (Murthy *et al*., 2002). Furthermore, the likely importance of the *cis* peptide bonds has been discussed (Weiss *et al*., 1998; Jabs *et al*., 1999). However, no further details or comprehensive suggestions have been advanced to date that could accommodate all of these observations, and the presumed key role for Ca^{2+} ions.

Close inspection of the structural changes of the TGase 3 enzyme activated by Ca^{2+} ion binding reveals that the channel opening is conical in shape extending from the surface of the protein toward the catalytic triad. The opening of the channel exposes side chains of Trp236 and Trp327 on the upper and outer surface of the channel (Ahvazi *et al*., 2002). This would seem to be a key step in 'opening' of the enzyme for reaction. We have also documented that

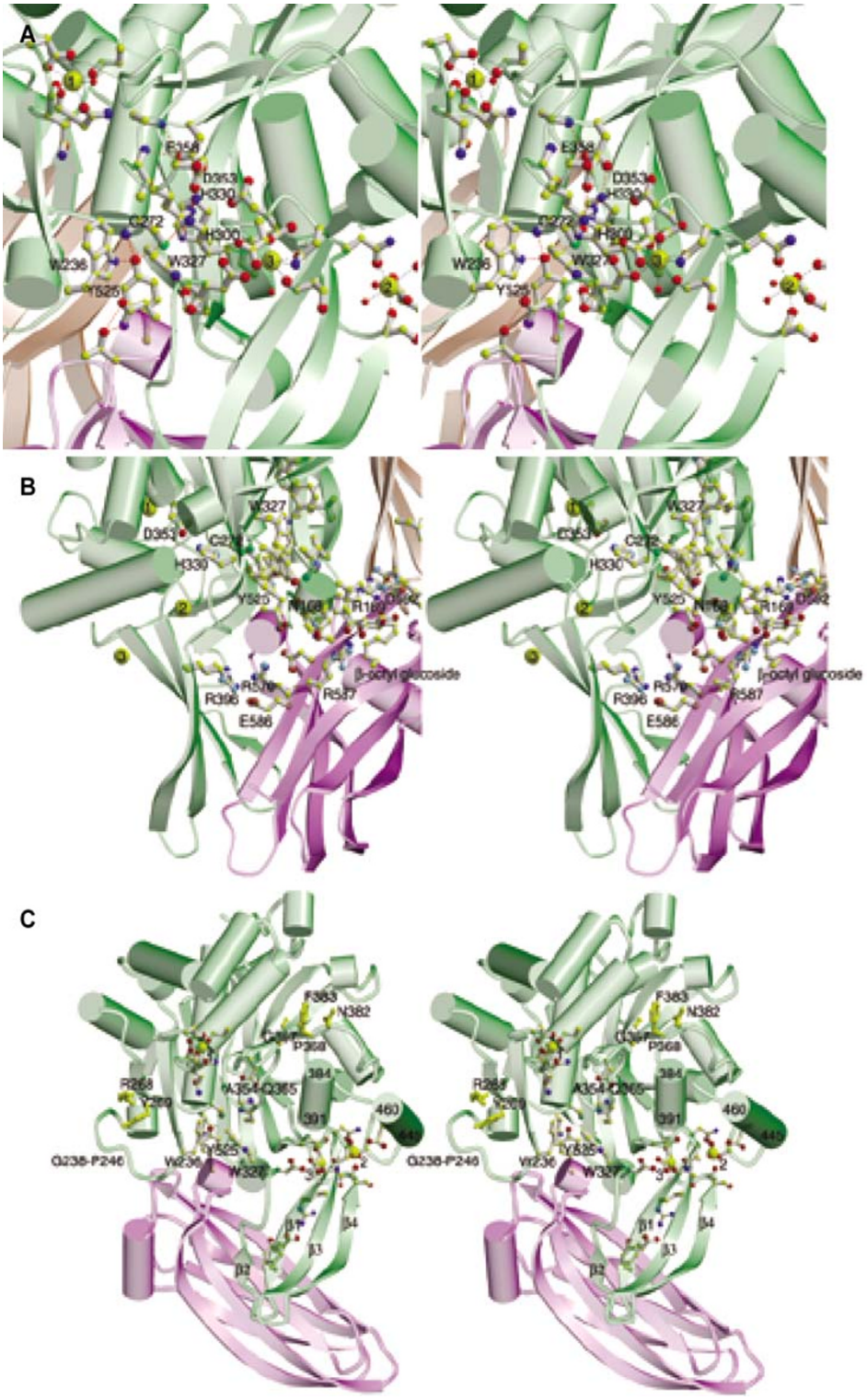

Figure 2. Stereo views of the active site region of activated TGase 3. (A) 'Front'. Shown are the side chain atoms of the catalytic triad residues Cys272, His330, and Asp353, coordination of the three Ca^{2+} ions, and hydrogen bonding of Tyr525, Trp236 and Trp327. (B) The β-octylglucoside molecule pocket on the 'back' side. The positions of side chains of some key residues of the zymogen (dark blue, dark red) are superimposed on the activated forms (light blue, pink). The β-octylglucoside molecule is bound in the hydrophobic pocket nearby. (C) The flap motif on the 'front' side of activated TGase 3 consists of four β-strands bound together with four α-helices by the catalytic Ca^{2+} ions in sites 2 and 3. The two non-proline *cis* peptide bonds Arg268-Tyr269, Asn383-Phe384, and Gly367-Pro368 *cis* peptide is shown in yellow color.

a channel closing appears on binding of Mg^{2+} ion at site three (Ahvazi *et al.*, 2003). This occurs because of movement of the loop following the β strand of residues Asp320 to Ser325, so that highly conserved residue Asp324 could not coordinate with the Mg^{2+} ion at site three. This movement closes the front of an existing channel on the surface of the enzyme. We have therefore wondered if the two sides of this

channel could serve as 'ports of entry' for the two substrates. The channel is about 16 Å deep (from the bulk solvent at the back side to the active site Cys272 residue) but only 13 Å wide at its widest point. Also, the side chains of a number of residues, in particular the guanidinium groups of Arg396, Arg420 and Arg570, protrude into the channel's cavity (Figure 2b). Accordingly, these insights impose severe constraints on how this cavity/channel could be used by substrates (Ahvazi *et al*., 2003). Therefore it is difficult to see how the 'back' entrance of the channel could be utilized for substrate access by the enzyme. Furthermore, there is another hydrophobic pocket that could not be used by substrate because a β-octylglucoside molecule that was incorporated into the crystallization condition lies in this pocket adjacent to the 'back' entrance (Figure 2b). Interestingly, this pocket is occupied by GDP nucleotide in the structure of TGase 2 (Liu *et al*., 2002), thus both substrates should approach the enzyme from the front side.

The function of *cis* peptide bonds in TGase 3

Non-proline *cis* peptide bonds are very rare, occurring in only 0.03% of the peptide bonds in solved protein structures (Weiss *et al*., 1998; Jabs *et al*., 1999). In TGase 3 structures (Ahvazi *et al*., 2002; 2003), we identified three peptide bonds that have *cis* conformations (Figure 2c). Two are non-proline *cis* peptide bonds. One is located at Arg268-Tyr269 in the β strand and the loop just prior to the α-helix that contains the active site Cys272 residue. The second occurs at Asn383-Phe384 in a loop adjoining two α-helices of the core domain located above the active site region. In addition, Gly367-Pro368 is a proline *cis* peptide bond and it is located on a strand adjacent to the Asn383-Phe384 *cis* peptide bond. These bonds are also present in the same locations in the other solved TGase structures, and sequence alignments reveal that their locations and flanking sequences have been highly conserved throughout the TGase family. All the residues on the amino-terminal side of the Arg268-Tyr269 bond are either charged or polar with long side chains. On the carboxy-terminal side, aromatic residues are usually present, except for a Cys residue in human TGase 4 (Grant *et al*., 1994). The enzymatically inactive P4.2 isoform has an aromatic residue (Sung *et al*., 1990) at the amino-terminal and negatively charged residues at the carboxy-terminal sides. The essential catalytic active site Cys-272 residue is located just downstream of Arg268-Tyr269, which explains the high degree of conservation in the enzymatically active isoforms. The residues on the amino-terminal side of the Asn382-Phe383 bond have mostly long polar side chains. On the carboxy-terminal side, most are aromatic residues, with the single exception in the P4.2 protein, which contains a proline residue instead at the equivalent position. Furthermore, the Gly367-Pro368 *cis*-peptide bond seems to have been retained in all TGases because of the absolute conservation of the sequence Gly-Pro. Altogether, these homologies suggest that the three *cis* peptide bonds are important for the stabilization, activation and/or mechanism of action of the enzymes (Weiss *et al*., 1998).

The stabilization of energetically unfavored *cis* peptide bonds in proteins is attributed to extensive hydrogen bonding as well as hydrophobic side chain interactions with neighboring residues (Jabs *et al*., 1999). Inspection of the extant structural data reveals that the *cis* bonds in TGase 3 and other isoforms are indeed tightly bonded in their local neighborhoods. In TGase 3, the Arg268-Tyr269 *cis* bond is involved in a web of hydrogen bonding. The hydroxyl side chain of Tyr269 forms a hydrogen bond with the main chain carbonyl oxygen of Thr241, located half-way along the loop Gly238-Asp245. This loop abuts Trp236, thought to be a key residue in the reaction mechanism of TGases. Also, the amide side chain of Asn235 is within the hydrogen bonding of the main chain nitrogen and hydroxyl side chain of Ser237. In addition, the carbonyl oxygen of the main chain of Tyr269 forms two hydrogen bonds: one with the indole nitrogen of Trp249 located on the short α-helical segment which follows the Gly238-Asp245 loop; and the other with the main chain nitrogen of the adjacent Gly270. Gly270 is further stabilized with the neighboring carbonyl oxygen of the main chain of Ala233, located on a β-strand that is situated between the Arg268-Tyr269 *cis* peptide bond and the Ca^{2+} ion at site 1. There is additional hydrogen bonding between the main chain carbonyl oxygen and nitrogen of Leu232 with the main chain nitrogen of Ser225 and the carbonyl oxygen of Ile223 respectively. Tyr269 is buried in the hydrophobic interior of the catalytic core domain. Together, the hydrogen bonding data suggest this *cis* peptide bond knits together a local cluster of β-strand, α-helix and loop motifs that adjoin Trp236. As it is just three residues from the active site Cys272 (in the zymogen ϕ = -45.8, ψ = -43.8 and in the activated form ϕ = -73.6, ψ = -54.0), it seems likely it could have a direct role in the enzyme reaction.

The other *cis* peptide bonds are likewise involved in a complex network of interactions and with each other (Figure 2c). The longest loop of TGase 3 (Ala354-Gln365) is located near the front surface of the enzyme and connects two antiparallel β-strand motifs that cover the top portion of Trp327, also thought to be critically involved in the enzyme reaction (Yee *et al*., 1994; Murthy *et al*., 2002). This loop is flanked

at one end by the catalytic triad residue Asp353 and Phe364 at the beginning of a β-strand containing the Gly367-Pro368 *cis* peptide bond. We note that the α-helical segment Asp384-Glu391 is preceded by a loop of residues that include the unusual Asn382-Phe383 *cis* peptide bond that has been conserved in TGases. This is involved in many interactions with neighboring residues that are also conserved in TGases. The amide side chain of Asn382 forms a hydrogen bond via a water molecule to the carbonyl side-chain oxygen of Asp341 and is hydrogen bonded to the guanidinium group of Arg339, which itself is stabilized by hydrogen bonding to Asp341. The main chain nitrogen of Phe383 is stabilized via the main chain carbonyl oxygen of Asn382, and is further hydrogen bonded to the main chain oxygen of Pro368 of the Gly367-Pro368 *cis* peptide bond. In this way, the Asn382-Phe383 non-proline *cis* peptide bond is firmly stabilized by the neighboring Gly367-Pro368 *cis*-peptide bond. In addition, the Asp353 side chain is within hydrogen bond distance of Ala354 and Thr355 respectively. Thus this complex network of bonds may effectively knit together the two *cis* peptide bonds, the catalytic triad residues and the surface loop Ala354-Gln365. One possible function of these associations therefore is to hold the active site residues in a fixed orientation for substrate access.

Thus while *cis* peptide bonds are energetically unfavorable, those in the TGases are unusually tightly bonded together with neighboring structural motifs. However, the precise role of these *cis* peptides remains unknown. One possibility is that one or more of them undergo a reversible *cis*→*trans* isomerization during the enzyme reaction cycle either spontaneously upon approach of a substrate or by protonation or nucleophilic attack on the carbonyl carbon of the peptide bonds. It has been suggested that metastable *cis* bonds could store potential energy to drive biochemical reactions in enzymes (Stoddard *et al*., 1998). However, if either of the bond(s) in TGases does reversibly isomerize, then the energy necessary to return from the *trans* to the metastable *cis* form to prepare for the next enzyme reaction cycle must be accounted for. On the other hand, isomerization is typically thought to be very slow and therefore instead may occur only once to activate an enzyme (Lin *et al*., 1993). Indeed, it has been suggested that a one-time *cis*→*trans* isomerization of the equivalent of the Asn382-Phe383 bond might be involved in fXIIIa activation (Weiss *et al*., 1998). Finally, there is an alternative possibility: the bonds may not isomerize during an enzyme activation step of the enzyme reaction cycle, but remain in the *cis* conformation to stabilize or anchor nearby motifs on the enzyme that do move during the reaction cycle. Primarily because of uncertainties about the energy of the reaction cycle (see below), our new model takes no position on isomerization.

The role of Ca^{2+} ion at site one

The Ca^{2+} ion in site one is located about 13 Å from the Cα atom of Arg268-Tyr269 *cis* peptide bond (Figure 2c). The ion is extraordinarily difficult to dislodge from the zymogen, and its binding affinity is K_d = 0.3 M (ΔH = -6.7±0.52 kcal/mol; Ahvazi *et al*., 2003), which suggests it is important for stabilization of the zymogen and activated enzyme forms. The Ca^{2+} ion in this site is heptacoordinated (distorted pentagonal bipyramid) by forming direct contacts with the main chain carbonyl oxygen atoms of Ala221, Asn224, Asn226, the carbonyl side-chains oxygen of Asn224, Asp228, and a water molecule. The loop Ile223-Val231 containing Asn229 has shifted away and Asp228 instead coordinates with the Ca^{2+} ion. In the activated TGase 3, the Ca^{2+} ion is shielded by the carbonyl side-chain oxygen of Asp228 located in a tight turn between β strands and α helix, and is an outlier in the Ramachandran plot (Ahvazi *et al*., 2002). The Asp228 side chain in the zymogen form (φ = 47.6, ψ = 40.6) is exposed while in the activated form it is buried (φ = -144.9, ψ = 19.7).

Five of the residues that coordinate with the ion are located on the loop segment that precedes the β strand Val231-Asn235. Activation of TGase 3 involves a change from six to seven coordinations with the Ca^{2+} ion, including Asp228 instead of Asn229 in the zymogen, because the loop moves over to effectively bury the ion within the interior of the activated enzyme. As noted above, the β strand is tightly associated by way of several links to the Arg268-Tyr269 *cis* peptide bond. It is therefore likely the Ca^{2+} ion at site one collaborates with the nearby *cis* peptide bond to maintain the structural integrity of the interconnected loops, β strand and α helical motifs. More specifically, we propose that they might serve as anchors so that the combined β strand Val231-Trp236 and loop motif of residues Gly238 to Pro246 could move during the reaction cycle as it pivots around Gly230 and Pro246. Movement of these residues would allow access of the Gln substrate into the vicinity of the active site (see below).

The role of Ca^{2+} ions at site two and three

Figure 2c reveals that the Ca^{2+} ion in site two (K_d = 4 M with ΔH = -4.639±0.15 kcal/mol for both Ca^{2+} ions at site two and three; Ahvazi *et al*., 2002) binds together upper portions of two β-strands of the core

domain, designated β3 (Asp395-Asn403) and β4 (Lys-407-Ser415), with two α-helices Asp384-Glu391 prior to the β3 strand and Ser445-Lys460 preceding the β4 strand, by coordination with side chains. Likewise, a Ca^{2+} ion in site three binds together upper regions of strands β1 (Leu306-Asp313) and β2 (Asn317-Leu-319) which is adjacent to the mobile loop Asp320-Ser325. Also, these α-helices are locked together in a network of interactions: Glu378 is hydrogen bonded to Lys454; Gln380 is linked by van der Waals interactions to Lys458; and Asn393 is hydrogen bonded to Glu448. Further, α-helices comprising Ser370-Glu376, just prior to the Asn382-Phe383 *cis* peptide bond is hydrogen bonded to α-helices Ser445-Lys460 through Glu376 to Tyr441, and Tyr441 is stabilized through van der Waals interactions by the guanidinium side chain of Arg375 that stacks over the Tyr441 ring. Therefore, a plausible role of the two catalytic Ca^{2+} ions is to hold together the whole right hand front surface of the catalytic core domain involving multiple β-strands, α-helices and loops interconnected with the two *cis* peptide bonds, to form a flap motif. In addition, Asp324 on the mobile loop and adjoining β2 strand is another key residue coordinated with the Ca^{2+} ion in site three. Ser325 preceding Asp324 is hydrogen bonded to the indole nitrogen of Trp327 (Figure 2a). We predict therefore that the orientation of the side chain of Trp327 is directly tied to the flap motif. Furthermore, part of this motif overlays part of the β-barrel 1 domain and is connected to it by a web of hydrogen bonds. These are: Tyr312 (β1 strand of flap motif) to Glu586; Gly316 and Asn317 (β2 strand) to Thr519 and Trp521; Arg396 (β3 strand) to Glu586; and Trp409 and Asn411 (β4 strand) to Glu582. In contrast, there are more hydrogen bonding interactions between the β1-β4 cluster and the β-barrel 1 domain in the zymogen observed. Thus the interface between the flap motif and the β-barrel 1 domain has been loosened in activated TGase 3.

Evidence that Ca^{2+} ions function to tighten structural motifs in TGase 3

We note that while changes in atomic coordinates between the zymogen (one Ca^{2+} ion at site one) and activated (full occupancy of Ca^{2+} ions at sites one,

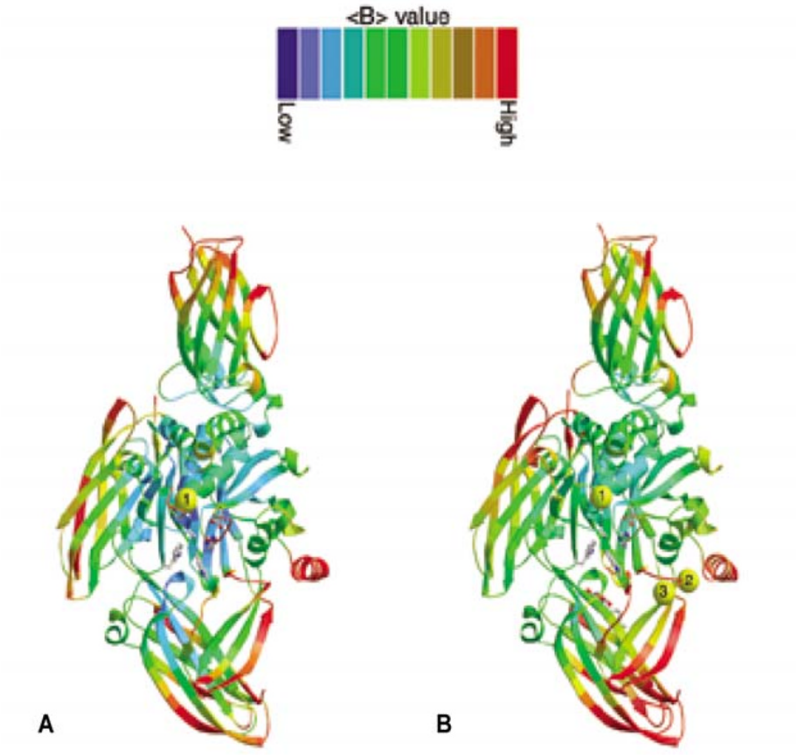

Figure 3. The ribbon diagram of solved structures of human TGase 3 enzyme based on temperature factor (Å^2) $<B>$ values from blue (low mobility) to red (high): (A) zymogen at 2.2 Å resolution. (B) The activated TGase 3 at 2.1 Å resolution. The β-octylglucoside is present in the core domain/β-barrel 1 domain interface near the 'back'.

two and three) forms of TGase 3 are small, superposition of the $<B>$ factors of the Cα backbones reveals that those of the activated form are sharply reduced. This suggests that flexibility of protein loops have been reduced upon Ca^{2+} ion chelation. Furthermore, this reduction in flexibility occurred at sites distant from the local environment of the Ca^{2+} ions (Figure 3). Accordingly, we propose that the Ca^{2+} ions not only serve to anchor large clusters of motifs on the enzyme's surface in conjunction with the *cis* peptide bonds, but also serve as anchor points to stabilize and tighten structural motifs, especially around the active site.

We searched the PDB database (at http://www.rcsb.org/pdb) to find other examples in which: (1) a protein has other domains aside from the Ca^{2+} or other metal ion binding site(s); (2) the conformational change upon metal ion binding is small; (3) the protein/enzyme is metal ion-dependent; (4) the crystal structures of both apo and metal ion bound forms have been determined at a similar resolution; and (5) the apo and metal ion bound crystal forms have the same crystal packing. In addition to TGase 3 there are several other proteins which fit some of these criteria and which display large changes in $<B>$ factors. We cite two examples. The first is des (1-52) grancalcin, which belongs to the Penta-EF family. Changes of $<B>$ factors between the apo form determined at 1.9 Å resolution (PDB 1K95) and the Ca^{2+}-bound form determined at 1.7 Å resolution (PDB 1K94) were observed (Jia *et al*., 2001). The Ca^{2+}-bound form has a very small conformational change (overall rms deviation of 0.53 Å, but $<B>$ factors of many residues are clearly changed. It has been shown that grancalcin exists as a homodimer, regardless of Ca^{2+} loading, and binds two Ca^{2+} ions per monomer with positive cooperativity. Interestingly, $<B>$ factors of the Ca^{2+} environment of the molecule are sharply reduced. Grancalcin binds secretory vesicles in a positive Ca^{2+}-dependent manner and binds L-plastin in a negative Ca^{2+}-dependent manner (Lollike *et al*., 2001). Crystal structures of des (1-52) grancalcin revealed that Ca^{2+}-dependent conformational change is very small, and it is thought that the N-terminal Gly/Pro-rich region is required for the conformational change. A second example, copper enzyme phenylethylamine oxidase (PDB 1AV4), also shows a drastic reduction of $<B>$ factors in almost all of its residues upon Cu^{2+} and topaquinone cofactor binding (Wilce *et al*., 1997). The structures of the apo- and holoenzymes used for comparison have both been determined at 2.2 Å resolution, and the overall rms difference between them is 0.4 Å These examples indicate that local ligand binding can change the $<B>$ factors of the residues distant from the binding sites. The Ca^{2+}-dependent flexibility changes of the surface residues of TGase 3 might, therefore, control the interaction with targets or self-assembly, instead of a large conformational change.

The active site of TGase 3

Sequence alignment of TGase isoforms reveals high similarity in residues that define a buried hydrophobic pocket around the active site Cys272, His330 and Asp353 triad residues. Superposition of the Cα backbone of solved structures of TGase 3, with other TGase 3 family and cysteine proteases reveals that the side chains of these residues are identical which is to be expected for their common reactions. Also, these positions resemble structurally unrelated but mechanistically similar enzymes such as papain (Schroder *et al*., 1993) which deamidate glutamines (Figure 4a). In TGase 3, Trp236, Trp273, Phe275, Trp327, Val331, Phe329, Trp332, Leu352, Thr355, Phe387, and Tyr525 residues wall the active site pocket environment.

Based on biochemical data (Lorand, and Conrad, 1984; Folk and Chung, 1985) as well as interpretations of TGase 3 structures (Ahvazi *et al*., 2002; Ahvazi *et al*., 2003), it is thought that the -SH group of Cys272 (using TGase 3 residue numbers) forms a thiolate-imidazolium ion pair with His330 with thiolate acting as the attacking nucleophile (Pedersen *et al*., 1994). The imino group of the His330 ring forms a hydrogen bond with the terminal oxygen atom of Asp353. On approach of a suitable Q* residue, an oxyanion intermediate is formed with Trp236, which then breaks down to release NH_3 and form a thiol-acyl intermediate. This is attacked by the ε-NH_2 of a K* substrate to form another tetrahedral oxyanion intermediate with Trp327, which in turns yields the cross-linked product. However, Tyr525 forms a hydrogen bond with Cys272 that must be broken to allow the reaction to proceed. Thus the question arises as to how the Q* substrate approaches the enzyme. One proposal for hfXIIIa and fTG is that the Q* substrate approaches the enzyme by displacing either β-barrel 1 or 2 or both, thereby displacing Tyr525 to expose and engage the active site (Yee *et al*., 1994; Noguchi *et al*., 2001). It was further proposed that a *cis*→*trans* isomerization of the non-proline *cis* peptide bonds might participate in this process (Weiss *et al*., 1998). Our new model proposes that substrates approach TGase 3 in a different way.

Molecular modeling offers important clues on how preferred substrates may dock

Molecular modeling studies can serve as a supple-

mentary tool in understanding how peptide chains bearing reactive Gln* and Lys* residues may complex with TGase 3. Interest is focused on the use of modeling because it can predict the inclusion modes, the stoichiometry of the complex, and the relative complexing efficiency. Commercially available software utilizes molecular mechanics and dynamic simulations. Docking programs have also been used for qualitative purposes. In this study herein, two docking methods for modeling TGase 3 complexation were evaluated. The SYBYL 6.7 program (Tripos Assoc. Inc., St. Louis, MO) offers two different methods, DOCK and FlexiDock, for docking the molecules into a binding site. These methods can be used to calculate the energy of the complex formed between the peptides and TGase 3 enzyme. Our model is predicated on the assumption that the front side of the enzyme, especially around the front of the channel, is utilized by the substrates. In addition, structural comparisons with the thiol protease papain are instructive since TGases and papain both utilize similar catalytic triad residues (Figure 4a).

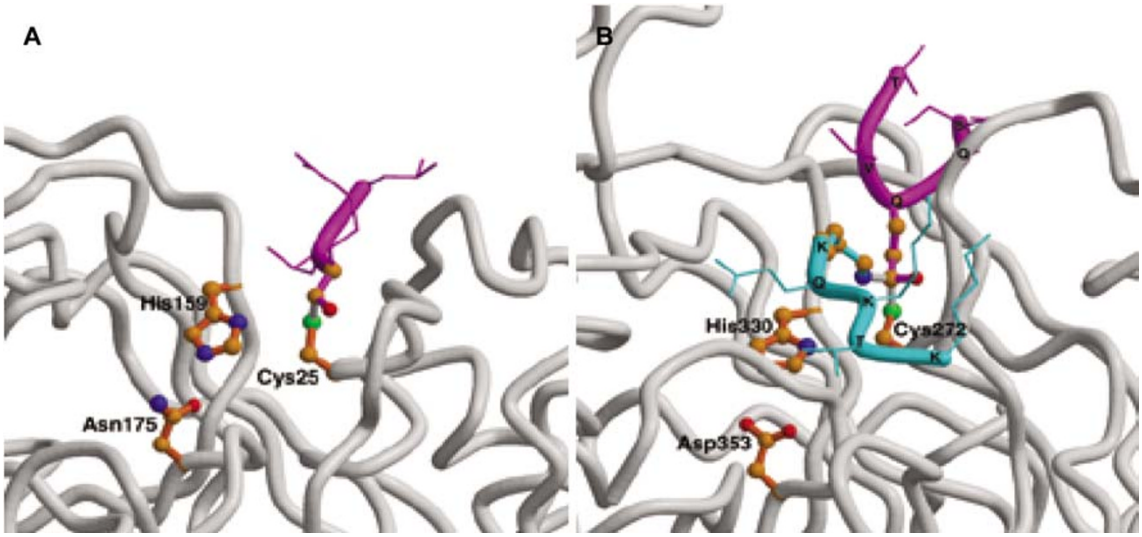

Figure 4. Schematic representations of crystal structure of: (A) papain (*gray*) with substrate leupeptin (*magenta*). B. The core domain of TGase 3 (*gray*) with the tetrahedrally coordinated intermediate formed by Cys272 and two peptide substrates (*cyan and magenta*).

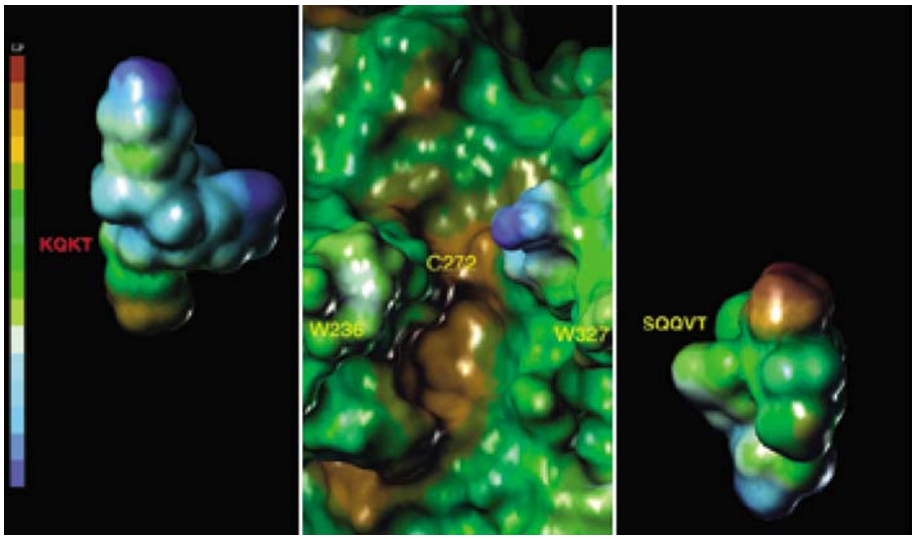

Figure 5. The mapping surface property of lipophilic (brown) and hydrophilic (blue) of TGase 3 enzyme active site is presented. The surfaces of both peptides SQQ*VT (from loricrin) for the Gln* substrate and KTKQK* (from small proline rich protein 1) as the Lys* substrate of TGase 3 enzyme also are shown.

We have modeled the peptides SQQ*VT (from loricrin) for the Gln* substrate and KTKQK* (from small proline rich protein 1) as the Lys* substrate, as these are used efficiently by TGase 3 (Candi *et al*., 1999). In TGases, the Gln* substrate forms a covalently bound tetrahedrally coordinated intermediate. The stereochemistry of this intermediate, the location of His330 and the limited pocket in the active site dictate that the Lys* substrate should occupy the same site as water in papain (Figure 4b). The resulting structure was minimized and further optimized by performing simulated annealing. Optimized conformations for the highly rigid SQQ*VT and KTKQK* were obtained using SYBYL 6.7 for use as substrate peptide molecules in the docking modules. They were placed inside the TGase 3 cavity pocket and FlexiDock was used to generate multiple conformations of them inside the pocket (Figure 5). Default parameters were used for both peptides, with iterations set to 20,000. A further increase in iterations did not show a significant change in the binding energy and conformation. FlexiDock scored all the orientations and calculated the binding energy for each orientation. This protocol generated the top 20 conformations and the difference in binding energy for these complexes was found to be <1 kcal/mol. DOCK was performed in a similar way by placing the peptide substrates inside the pocket. The Docking software performed by

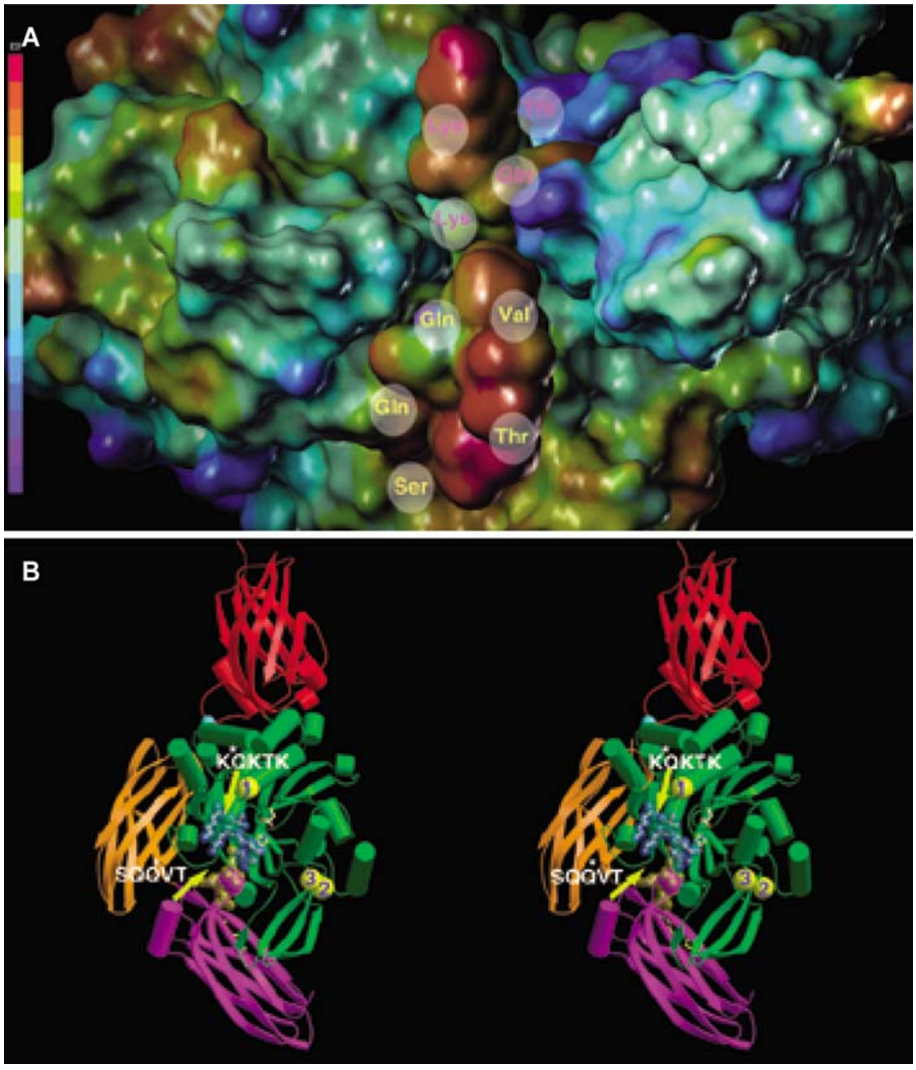

Figure 6. (A) A view of electrostatic potential surface property docking of SQQ*VT and KTKQK* into the binding active site using FlexiDock. The top of the electrostatic potential property ramp (red) is the most positive and the bottom of the ramp (purple) is the most negative. (B) Stereo view of the Q* and K* substrates determined by molecular docking. The side chains of the catalytic triad residues as well as the Q* and K* residues are shown in ball-and-stick. As the loop Ile523-Asn526 harboring Tyr525 occupies space where the VT residues of the substrate should reside, we propose that the Q* substrate displaces this loop.

changing their orientation such that their lipophilic portions were inserted into the TGase 3 cavity pocket. The dock complex by holding TGase 3 rigid was subsequently minimized until an energy gradient of 0.01 kcal/mol was reached. Changing the starting orientation of the peptides produced similar results, suggesting a unique binding mode for these peptide ligands (Figure 6a).

The energy scores obtained from DOCK showed an inverse correlation, while FlexiDock energy values showed no correlation with the stability constants. DOCK and FlexiDock scores suggest a favorable interaction between peptides and TGase 3, evident from the negative binding energies, making it possible to predict the formation of a stable 1:1 complex, although no information on dynamic behavior can be obtained. These methods do not take into account the hydrophilichydrophobic interactions or the solvent role in complexation, hence they are merely of qualitative importance.

The docking of the peptide substrates inside the TGase 3 cavity obtained using DOCK and FlexiDock are shown in Figure 6a and b, respectively. For these complexes, it seems that hydrophobic interactions are more important than the electrostatic and hydrogen bonding interactions. This is also apparent by visual inspection of the docked peptides as evident by insertion of the lipophilic portions of the molecule inside the TGase 3 cavity pocket.

Table 1. Tabulated drawing of the key residues involved in the two parts of the TGase reaction. The 16 residues in the vicinity of the active site pocket have been divided into three groups; those, which define the glutamine, pocket and the lysine, pocket, or are common to the vicinity. Residues that are different and/or make different interactions, conserved residues, and the common tryptophan residue involved in oxyanion formation are listed separately.

Transglutaminase	TGase 3	fXIIIa	fTG	TG2
		Glutamine pocket		
Different residues	Arg247	Ser290	Tyr247	Met252
	Asp566	Glu601	His566	Asn559
	Phe329	Tyr372	Phe331	Phe334
Conserved residues	Gln271	Gln313	Gln271	Gln276
	Trp273	Trp315	Trp272	Trp278
	Trp327	Trp330	Trp329	Trp332
	Asn328	Asn371	Asn330	Asn333
Oxyanion intermediate	Trp236	Trp279	Trp236	Trp241
		Lysine pocket		
Different residues in	His300	His342	His300	His305
hydrogen bonding	Glu358	Glu401	Glu360	Glu363
	Glu391	Glu434	Glu393	Glu396
Conserved residues	Trp327	Trp370	Trp329	Trp332
	Phe275	Phe317	Phe275	Phe280
	Pro356	Pro399	Pro358	Pro361
Oxyanion intermediate	Trp236	Trp279	Trp236	Trp332
		Conserved active site pocket residues		
	Val331	Cys374	Cys333	Cys336
	Trp332	Trp375	Trp334	Trp337
	Leu352	Leu395	Leu354	Leu357
	Thr355	Thr398	Thr357	Thr360
	Phe387	Phe430	Phe389	Phe392
	Tyr525	Tyr560	Tyr515	Tyr516

Glutamine Pocket

Thus the SQQ*VT substrate should approach from the 'front' side of TGase 3 from direction of β-barrel 1 and 2 domains. In order for the substrate to reach Cys272, the substrate is required to move under the loop Gly238-Pro246 and by displacing it, the access to Trp236 will be achieved (Figure 2c). The moving of this loop could be accomplished from the stored potential energy of Ca^{2+} ion binding at site 1. This movement of the loop region results in forming the thiol intermediate/oxyanion complex with Trp236. Breaking the hydrogen bonding with Tyr525 allows to displace outward only a small loop of sequences bearing Tyr525 on the β-barrel 1 domain. This would result in loss of the hydrogen bond between the Tyr525 and Cys272 residues, and exposure of the catalytic Cys residue at the active site. Then substrate, even a bulky substrate, could penetrate the hydrophobic channel to the 'front' side of the enzyme allowing the large portion of protein on the surface of TGase 3 enzyme. This movement can only occur after "unlocking" the enzyme by proteolytic cleavage at Ser469. In TGase 3, the resultant exposed hydrophobic pocket is lined at the front and interior by the residues Arg247, Phe329, Trp236, Gln271, Trp273, Trp327 and Asn328 (Table 1). However, there are key substitutions and resultant important differences in residue interactions with respect to the other TGase enzymes: Arg247 is a tyrosine in fTG and a serine in fXIIIa, and Phe329 is conserved in fTG but is replaced by Tyr372 in fXIIIa. In TGase 3, Arg247 forms a hydrogen bond with the carbonyl side chain of Asp566 located in the β-barrel 2 domain. In fXIIIa, the hydroxyl side chain of Tyr372 instead forms a hydrogen bond with the equivalent Glu601. In fTG, the side chain of Tyr247 is pointed in a different direction so that no stabilizing hydrogen bonds are formed. In addition, Val331 in TGase 3 is notably different from the fXIIIa and fTG enzymes which each have a Cys residue instead at equivalent position. In those two enzymes, loss of the hydrogen bond between their respective Tyr and active site Cys residues could allow the formation of a disulfide bond that would inhibit the respective enzymes (Noguchi *et al*., 2001). It has been suggested therefore that the approach of the glutamine substrate should break this proposed disulfide bond to activate the enzyme (Yee *et al*., 1994; Noguchi *et al*., 2001). If so, then the TGase 3 enzyme is different in the sense that once proteolytically activated at Ser469, it is constitutively functional. Accordingly, Val331 residue is then a key residue that might govern enzyme activity in the context of the glutamine substrate.

Finally, the available TGase structures demonstrate that Trp236 (Trp279 in fXIIIa, Trp236 in fTG) is well

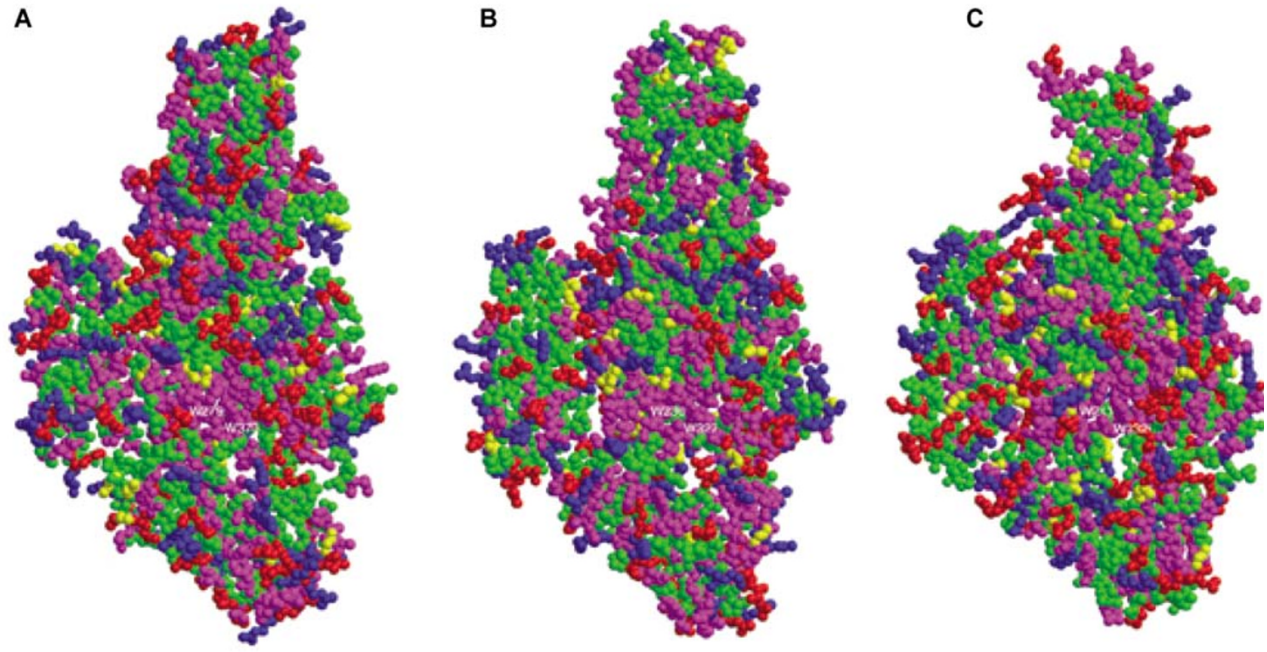

Figure 7. The figure represents a comparison view of CPK surface of the a) human fXIIIa, b) TGase 3, and c) TGase 2 enzyme structures. In this presentation, hydrophobic residues (Y, F, L, IL, V, P and A) are colored in green while charged residues are colored in blue (K and R) and red (D and E), respectively. All other residues are polar and colored in magenta except residue (G) in yellow.

placed to stabilize the tetrahedral oxyanion intermediate of the thioacyl enzyme form by hydrogen bonding through the indole nitrogen atom of Trp236, and the main chain nitrogen atom of Cys272. The χ_2 for the side chain of Trp236 in TGase 3 is 96.0° which is similar to the value of -105.0° for the equivalent Trp279 in fXIIIa. However, the orientation of the Trp236 ring in the fTG enzyme is +93.0°. These values strongly suggest that the angle of approach of the glutamine substrate is similar for TGase 3 and fXIIIa, but quite different for fTG. Figure 7 shows the comparison of the charge distribution in the region of the proposed glutamine substrate-binding site. Such differences in the charge distribution may account for the different substrate specificity within the TGase family. In summary therefore, the analyses based on molecular modeling and the inspection of the structures suggest that Arg247, Phe329, Val331 and Asp566 of TGase 3 represent four key residues that may confer substrate specificity and warrant further detailed study.

Lysine Pocket

Although the degree of movement of the flap region is not known, the mobility data suggest that the few hydrogen bonds anchoring it to the underlying strands of the β-barrel 1 domain can be broken. This results in the formation of an enlarged pocket that would allow the approach of the K* substrate in figure 6a and b. The non-catalytic His300 residue, and residues Trp327, Phe275, Glu358 and Glu391 bound the lysine pocket. Obviously, the K* substrate has to approach the oxyanion intermediate from a direction different from that occupied by the Q* substrate. Molecular docking shows that KTKQK* can only approach the thioacyl intermediate on the opposite side of the channel about 120° with respect to the Q* substrate, from the 'front' side of the enzyme, by passing over the Ca^{2+} ion at site one and finally to the 'left' side of the longest loop within TGase 3 (Ala354-Gln365) that is located near the front surface of the enzyme which spans between two antiparallel β-strand motifs (Figure 2c). In both solved TGase 3 structures, His300 is hydrogen bonded to Glu358. Interestingly, there is a notable difference with hfXIIIa. The equivalent residue (His342) is hydrogen bonded to a different residue (Glu434) instead, which thereby shifts the orientation of the imino groups of His300. In the case of the fTG enzyme, the equivalent His300 residue does not make similar interactions. Thus, the X-ray structures indicate that the orientation of the His300/Glu358 diad and charge distribution on the surface in TGase 3 could contribute specificity to the recognition of the K* substrate (Figures 6a, 7). This diad deprotonizes and correctly orients the K* side chain within the pocket for reaction with the thioacyl intermediate (Yee *et al.,* 1994; Pedersen *et al.,* 1999). In particular, Trp327 of TGase 3 is located opposite Trp236 involved in oxyanion formation (Yee *et al.,* 1994). In this way, as in all TGase enzymes, the aliphatic part of the K* side chain is sandwiched between their exposed side chains. These residues thereby provide both the tetrahedral oxyanion intermediate and stability for both the Q* and K* substrates. Finally, the cross-linked product diffuses away from the TGase 3 surface.

Features of Model

Molecular modeling studies were conducted and the inclusion modes of the TGase 3 enzyme with the peptides SQQ*VT (from loricrin) for the Gln* substrate and KTKQK* (from small proline rich protein 1) as the Lys* substrate were determined. The molecular modeling and the energy of the calcium sites show that the model of TGase 3 enzyme reaction is energetically economical. Docking programs were successfully used to study the inclusion of two peptide molecules in the TGase 3 enzyme cavity. In this particular case, a better understanding of the various interactions was obtained by studying these complexes using several methods. The flexible flap motif has a total buried surface area of 797 $Å^2$ with the β-barrel 1 domain, and an additional 600 $Å^2$ are needed to break the Tyr525 hydrogen with Cys272 and Trp236. In contrast, an earlier model had proposed that the Q* substrate approaches the active site by displacing either or both the β-barrel 1 and 2 domains including movement of the conserved Tyr525 residue to allow access to the active site (Yee *et al*., 1994). This requires interruption of at least 3000 $Å^2$ of interdomainal surfaces (Weiss *et al*., 1998). The model proposes herein that the initial binding of the two Ca^{2+} ions transform a naïve enzyme to the active form on approach of the first Q* substrate. The model requires that the energy needed for subsequent reaction cycles should be much less than for a naïve enzyme, and may be contributed by the favorable binding energy of a specific Q* substrate, or by residual bonding energy from the release of NH_3 from the previous reaction cycle. The model predicts for the first time the role of Ca^{2+} ions, in TGase 3, by the creation of a channel and by stabilizing and contributing energy that regulates access of Q* substrates into the active site.

Based on detailed analyses of current structural information on the zymogen and the activated forms of TGase 3, as well as molecular modeling of Gln and Lys substrates, we present here a new model of TGase 3 for its mechanism of action. The model

can offer explanations for: 1) the absolute requirement for three Ca^{2+} ions; 2) the purpose of the channel; 3) the role of the three *cis* peptide bonds; 4) the energy needed to break key hydrogen bonds and move certain sequence motifs on the enzyme surface that occlude access to the active site region; 5) how the glutamine and then lysine substrates approach the active site triad residues to perform the chemistry of reaction; and 6) why the TGase reaction as currently understood is so slow. What is far from clear, at this time is an explanation for the known marked degree of substrate specificity of TGase 3. Nevertheless, we believe this model will stimulate efforts to generate experimental data for a better model, which may offer clues as to how TGase 3 specificity (and for other enzyme isoforms) is determined and whether it will be possible to design TGase 3 (and other) isoform specific inhibitors.

Figures

The figures were generated with Molscript (Kraulis, 1991), and Raster3D (Merritt and Bacon, 1997). The molecular surface property was characterized for topography and electrostatic potential distribution through MOLCAD (computer aided molecular design) modual implemented in Sybyl program. The visualization of spatial surface was performed through Connolly's program (designated as Connolly surfaces) in Sybyl 6.7 Tripos Assoc. Inc., St. Louis, MO.

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